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Cannabidiol provides long-lasting protection against the deleterious effects of inflammation in a viral model of multiple sclerosis: A role for A_{2A} receptors

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ABSTRACT

Inflammation in the central nervous system (CNS) is a complex process that involves a multitude of molecules and effectors, and it requires the transmigration of blood leukocytes across the blood–brain barrier (BBB) and the activation of resident immune cells. Cannabidiol (CBD), a non-psychotropic cannabinoid constituent of *Cannabis sativa*, has potent anti-inflammatory and immunosuppressive properties. Yet, how this compound modifies the deleterious effects of inflammation in TMEV-induced demyelinating disease (TMEV-IDD) remains unknown. Using this viral model of multiple sclerosis (MS), we demonstrate that CBD decreases the transmigration of blood leukocytes by downregulating the expression of vascular cell adhesion molecule-1 (VCAM-1), chemokines (CCL2 and CCL5) and the proinflammatory cytokine IL-1β, as well as by attenuating the activation of microglia. Moreover, CBD administration at the time of viral infection exerts long-lasting effects, ameliorating motor deficits in the chronic phase of the disease in conjunction with reduced microglial activation and pro-inflammatory cytokine production. Adenosine A_{2A} receptors participate in some of the anti-inflammatory effects of CBD, as the A_{2A} antagonist ZM241385 partially blocks the protective effects of CBD in the initial stages of inflammation. Together, our findings highlight the anti-inflammatory effects of CBD in this viral model of MS and demonstrate the significant therapeutic potential of this compound for the treatment of pathologies with an inflammatory component.

Introduction

MS is a chronic, inflammatory demyelinating disease of the CNS that is characterized pathologically by the presence of focal areas of inflammatory-mediated demyelination in the white matter of the brain and spinal cord (Trapp and Nave, 2008). The etiology of MS remains unknown, although several factors including genetic predisposition, viral infection and autoimmunity are thought to be involved. In the early stages of the disease, there is a recruitment of activated immune cells that promotes the adhesion and transmigration of leukocytes across the BBB (Archelos et al., 1999; Lee and Benveniste, 1999; Ransohoff, 1999). These events depend on a variety of interactions, including an initial transient contact between circulating leukocytes and the vascular endothelium, mediated by adhesion molecules, and the binding of leukocytes to chemotactic chemokines present on the endothelial surface. This latter event promotes the expression of integrins on the leukocyte surface and ultimately, it leads to diapedesis and leukocyte entry into the CNS (Engelhardt, 2008). It is widely recognized that enhanced leukocyte trafficking to the CNS is a key feature of MS (Lassmann, 2008). Therapies have been designed to target CNS inflammation, such as natalizumab (Tysabri®), a humanized antibody to very late antigen-4 (VLA-4: Krumbholz et al., 2012) that is essential for the homing of immune cells to the brain and other organs. Indeed, natalizumab is currently prescribed for the treatment of relapsing remitting MS (Steinman, 2012). Similarly, cannabis-based medicines containing tetrahydrocannabinol (THC) and CBD have also been approved for the treatment of pain and spasticity in MS (e.g., Sativex®). The promising therapeutic potential of cannabinoids (reviewed by Pertwee, 2012) is limited due to the central CB1 receptor-mediated psychotropic effects. CBD is the major *Cannabis* derived non-CB1/CB2 ligand that is devoid of psychotropic effects and even, inhibits many central effects of THC (Zuardi, 2008). Evidence shows that CBD acts as an immunomodulator (Mechoulam et al., 2007) and exhibits a wide range of anti-inflammatory properties

Abbreviations: BBB, blood brain barrier; CBD, cannabidiol; CCL2, chemokine ligand 2; CCL5, chemokine ligand 5; CCR2, chemokine receptor 2; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; THC, tetrahydrocannabinol; TMEV-IDD, Theiler's murine encephalomyelitis virus-induced demyelinating disease; VLA-4, very late antigen-4.

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including the inhibition of IL-6 and the activation of antiinflammatory pathways in microglial cells (Kozela et al., 2010). CBD has been shown to induce apoptosis of microglial cells through lipid raft involvement (Wu et al., 2012). Moreover, CBD ameliorates experimental autoimmune encephalomyelitis (EAE) symptomatology by diminishing inflammation, microglial activity and leukocyte infiltrates in the spinal cord (Kozela et al., 2011).

CBD has a broad spectrum of pharmacological actions (Izzo et al., 2009) including the inhibition of an equilibrative nucleoside transporter which in turn led to the increase of extracellular adenosine (Carrier et al., 2006). In acute injury models CBD diminishes inflammation through adenosine A_2 receptors (Ribeiro et al., 2012). Neuroprotective effects of CBD in hypoxic–ischemic brain damage also involve adenosine A_2 receptors (Castillo et al., 2010).

We previously reported that enhanced endocannabinoid tone and treatment with synthetic cannabinoid agonists improve motor behavior in TMEV-IDD mice, reducing infiltration into the spinal cord (Arevalo-Martin et al., 2003; Correa et al., 2005; Docagne et al., 2007; Ortega-Gutierrez et al., 2005). However, the effects of CBD in the induction phase of TMEV-IDD have not been previously described. In the present study, we investigated the effects of CBD in the initial stages of the inflammatory response in the TMEV-IDD experimental model of MS, and the impact of this treatment on the symptomatology and the neuroinflammation that characterizes the chronic phase of the disease. Finally, we investigated the contribution of adenosine A_{2A} receptors to these effects of CBD.

Materials and methods

Animals and Theiler's virus infection

TMEV-IDD-susceptible female SJL/J mice from our in-house colony (Cajal Institute, Madrid) were maintained on a 12 h light/dark cycle with ad libitum access to food and water. Four-week-old mice were inoculated intracerebrally in the right hemisphere with 2×10^6 plaque forming units (PFU) of the Daniels (DA) strain of TMEV in 30 μl of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), as described previously (Lledo et al., 1999), or with 30 μl of DMEM + 10% FCS in the case of sham (non-infected) operated mice. Animals were handled in accordance with the European Union animal care guidelines (2010/63/EU).

In vivo CBD and ZM241385 treatments

Sham or TMEV-IDD mice were administered with CBD (5 mg/kg, kindly provided by Dr. Mechoulam from the University of Jerusalem, Israel) intraperitoneally (i.p.) or the vehicle alone, once daily from days 1 to 7 post-infection (p.i.) for the analysis at induction and from days 1 to 10 p.i. to analyze the long-term effects. The duration of the treatment was chosen based on previous studies using the TMEV-IDD model (Docagne et al., 2007; Mestre et al., 2005; Ortega-Gutierrez et al., 2005). To identify the implication of A_{2A} receptors in the effects of CBD, the ZM241385 antagonist (5 mg/kg) was injected to control (sham), TMEV and CBD treated animals 30 min before treatment with the cannabinoid, according to the work of Ribeiro et al. (2012). Animals for the analysis at induction were sacrificed with an overdose of anesthetic (pentobarbital) 8 days p.i., while those treated for 10 days were maintained in the animal facility for 80 days p.i., when signs of disease were evident in the TMEV-infected mice. At this point, the motor activity was evaluated and the animals were sacrificed and processed for tissue collection.

Behavioral analysis: spontaneous motor activity

Locomotor activity was evaluated in mice using an activity cage (Activity Monitor System, Omnitech Electronics Inc., Columbus, OH, USA) coupled to a Digiscan Analyser. The number of times that the animals broke the horizontal or vertical sensor beams was measured in two 5-min sessions.

Tissue processing

Mice were anesthetized with pentobarbital (50 mg/kg body weight, i.p.) and perfused with saline. The animals' brain and spinal cord were fixed overnight in 4% paraformaldehyde prepared in 0.1 M phosphate buffer (PB) and cryoprotected in sucrose solution in 0.1 M PB (15% followed by a 30%). Coronal cryostat sections (30 μm thick) were then processed for immunohistochemistry.

Immunohistochemistry

Free-floating brain or spinal cord sections were washed three times for 10 min with 0.1 M PB and blocked for 1 h at room temperature in blocking buffer (0.1% Triton X-100 and 5% normal goat serum: Vector Laboratories, Burlingame, CA, USA) after inhibiting the endogenous peroxidase. The sections were then incubated overnight at 4 °C in blocking buffer containing the antibodies against VCAM-1 (1:1000; DSHB, USA) or Iba-1 (1:1000; Wako, Osaka, Japan). The following day, the sections were rinsed three times for 10 min with PB + 0.1% Triton X-100 and they were then incubated for 1 h with a biotinylated (Vector Laboratories, CA, USA) or Alexa Fluor-conjugated (Molecular Probes Inc, Eugene, OR, USA) goat anti-rabbit antibody. For immunofluorescence, the sections were then rinsed three times for 10 min with PB and mounted. For immunostaining with DAB, the sections were incubated for 1 h with a biotin-peroxidase complex (Vector Laboratories, CA, USA) and finally with the chromogen 3,3′ diaminobenzidine tetrahydrochloride (DAB: Sigma-Aldrich, St. Louis, MO, USA). After staining, the sections were dehydrated, cleared with xylene and coverslipped. In all cases, the specificity of staining was confirmed by omitting the primary antibody.

Microscopy and image analysis

Images were acquired for immunofluorescence on a Leica TCS SP5 confocal microscope and for immunohistochemistry with a Zeiss Axiocam high resolution digital color camera. Individual images of 4–5 sections were acquired from at least 6 animals per group. The immunostaining quantification was performed using ImageJ software (designed by National Institutes of Health) as detailed as follows: first, we defined the regions of interest (white matter in cervical spinal cord, whole area in cerebral cortex) with the freehand tool; second, we split the channels of the selected area, obtaining one image per channel; third, we established a constant threshold of intensity in sham animals to apply it to all the experimental groups; and four, we measured the staining intensity of all images within the experiments. The data are presented as the percentage of the total area that was stained with respect to sham animals.

Inflammatory infiltrate analysis

Slices were stained with hematoxylin and eosin (H&E) and the inflammatory infiltrate was evaluated by scoring the amount of infiltrates on a scale of 0–4 in specific brain areas (cortex and striatum) and in blood vessels. A score of 0 reflects the absence of infiltrates, 4 reflects the highest amount of infiltrates, while all the intermediate scores (1, 2 and 3) define the increasing density of infiltrates in the brain tissue. Representative images showing scale infiltrates 1–4 are presented in the Supplementary data.

Endothelial cell culture and stimulation with TMEV virus

Murine brain endothelial cells (b.end5) with brain endothelium-like properties were obtained from the European Collection of Cell Cultures (UK). The cells were plated in 12-well plates at a density of 10^5 cells/ml

and grown at 37 °C in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin, and in a humidified atmosphere of 5% CO_2. The cells were maintained in these conditions for 1 day and they were then switched to restricted growth conditions (1% FBS) 1 h before the experiments. CBD (Tocris, Bristol, UK) was added 1 h prior to infection of the cells with the DA strain of TMEV virus (2×10^5 PFU) and the cells were maintained in these conditions for 20 h. To identify the implication of A_{2A} receptors in the effects of CBD, the ZM241385 antagonist (0.1, 1 and 5 μM) was added 30 min before treatment. For ELISA assays of the sVCAM-1 adhesion molecule the supernatants of the cell cultures were collected.

Adhesion experiments

Brain endothelial cells were plated as described above at a density of 10^5 cells/ml and they were then infected with the DA strain of TMEV virus (2×10^5 PFU) for 4 h. Next, 2×10^5 leukocytes from the lymphatic nodes of experimental animals (vehicle or CBD-treated sham and TMEV animals, 8 days p.i.) were homogenized in cold phosphate buffer saline (PBS) with the plunger of a syringe, they were then filtered through a 70 μm cell strainer to obtain a single cell suspension, centrifuged for 5 min at 1200 rpm and resuspended in RPMI supplemented with 10 mM HEPES (pH 7.4), 2 mM glutamine, 10% FBS and β-mercaptoethanol (50 μM). The leukocytes were then stained with calcein acetoxymethyl ester (AM, 5 μM) and allowed to adhere to the endothelial monolayer for 20 h. Non-bound leukocytes were removed, and adhered leukocytes were counted in 5 fluorescence and phase contrast microphotographs/field using MetaMorph software, as described previously (Mestre et al., 2011).

Astrocyte cell culture and stimulation with IL-1β and TNFα

Rat astrocyte cultures were prepared from postnatal Wistar pups (0–2 days after birth) as described previously (Mecha et al., 2011). After isolation, astrocytes were grown in poly-D-lysine-coated 12-well sterile plates at a density of 5×10^5 cells/ml medium using DMEM supplemented with 10% horse serum (HS), 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. The medium was switched 24 h later to DMEM alone for 1 h, and the cells were then incubated for 6 h with IL-1β + TNFα (10 ng/ml each) and with CBD (1 or 5 μM, Tocris, Bristol, UK) or the vehicle alone (70% ethanol). The CCL-2 chemokine was assayed in the supernatants of the cell cultures by ELISA.

ELISA analysis

The CCL2 content in astrocyte cultures and the VCAM-1 content in endothelial cultures were measured by solid phase sandwich ELISA, using monoclonal antibodies specific for CCL2 and the soluble fraction of VCAM-1, respectively. The CCL2 and sVCAM-1 contents in the supernatants were quantified according to the manufacturer's instructions using rat-specific CCL2 (Biosource International, CA, USA) and mouse-specific sVCAM-1 ELISA kits (R&D Systems, MN, USA). The sensitivity of the assays was 4.7 pg/ml for CCL2 and 30 pg/ml for sVCAM-1.

Reverse transcription (RT) and real-time polymerase chain reaction (PCR)

Total RNA was extracted from nervous tissue (prefrontal cortex and spinal cords) or from cell cultures using RNeasy mini columns (Qiagen, UK). Genomic DNA contamination was avoided by DNase I degradation (Qiagen, UK) and the RNA yield was determined using a Nanodrop spectrophotometer (Nanodrop Technologies). Total RNA (1 μg in 20 μL) was reverse transcribed into cDNA using poly-dT primers and the Promega reverse transcription kit (Promega, Spain). Primers were (Applied Biosystems, UK) TNFα: sense (GACTCCCCCCTCCGTCTAAG) and antisense (CGCAGTAAAGCCCACGTTGT); Il-1β: sense (TGGTGTGTGACGTTCCCATT) and antisense (TCCATTGAGGTGGAGAGCTTTC); CCL2: sense (AGCAGG TGTCCCAAAGAAGCTGTA) and antisense (AAAGGTGCTGAAGACCTTA GGGCA); CCL5: sense (TCGTGCCCACGTCAAGGAGTATTT) and antisense (TCTTCTCTGGGTTGGCACACACTT); CCR2: sense (GGCCACCACACCGTAT GACT) and antisense (ACATGTTGCCCACAAACCAAAG); and 18S: sense (ATGCTCTTAGCTGAGTGTCCCG) and antisense (ATTCCTAGCTGCGGTAT CCAGG). SYBR® PCR was performed using 1 μL of cDNA (corresponding to 50 ng RNA input) in a Universal TaqMan Mastermix with 100 nM primers and 50 nM probe. Cycling conditions were as follows: 50 °C for 2 min; 95 °C for 10 min; and 40 amplification cycles of 95 °C for 15 s and 60 °C for 1 min. Samples were assayed using the Applied Biosystems PRISM 7500 sequence detection system, assaying each sample in triplicate and running a 6-point standard curve in parallel. To ensure the absence of contamination with genomic DNA, a control sample using RNA as the template was run for each set of extractions. Relative quantification was obtained by calculating the ratio between the values obtained for each gene of interest and those of the 18S house-keeping gene. The results are expressed as a percentage with respect to the sham animals for each time point.

Data analysis

All the data are expressed as the mean ± SEM. One-way ANOVA followed by the Bonferroni post-hoc test or Kruskal–Wallis ANOVA followed by Mann–Whitney U test was used to determine the statistical significance in all cases. The level of significance was set at $p \leq 0.05$.

Results

CBD decreases VCAM-1 and chemokine expression in vivo and in vitro

VCAM-1 is a member of the immunoglobulin supergene family that is mainly expressed by endothelial cells and that binds selectively to VLA-4 expressed by monocytes and lymphocytes (Engelhardt, 2008). A rapid increase in VCAM-1 expression was detected in the brains of TMEV-infected animals but not in sham animals (Fig. 1A), consistent with previous findings (Brosnan et al., 1995; Mestre et al., 2009). However, when mice were administered CBD for 7 consecutive days immediately after infection VCAM-1 expression remained at control levels in the brain, as revealed by immunohistochemistry (Fig. 1A). Indeed, ELISA analysis of sVCAM-1 in endothelial cells revealed that CBD completely blocked TMEV-induced release of this adhesion molecule (Fig. 1B; $p \leq 0.001$). This block in VCAM-1 expression was confirmed when in vitro cell adhesion was assessed. Accordingly, lymphocytes from TMEV-infected animals (8 days p.i.) exhibited increased adherence to a monolayer of endothelial cells previously infected with TMEV ($p \leq 0.001$), while those extracted from TMEV-infected mice treated with CBD did not adhere to endothelial stimulated cells to the same extent ($p \leq 0.001$).

The upregulation of the chemokines CCL2 and CCL5 in the spinal cord of TMEV-IDD mice has been associated with the development of the clinical disease (Hoffman et al., 1999). Thus, we investigated whether TMEV induces the expression of these molecules in the prefrontal cortex of infected mice. In the acute phase of infection with TMEV, we observed strong increases in the expression of CCL2, CCL5 and the CCR2 receptor mRNA (Fig. 2A). CBD treatment significantly reduced the expression of the CCL2 and CCL5 transcripts ($p \leq 0.05$), and it appeared to produce a slight but not significant decrease in CCR2 expression (p value = 0.460). The effect of CBD on chemokine induction was also analyzed using in vitro approaches (Fig. 2B). CBD treatment (1 and 5 μM) had no effect on basal CCL2 release in astrocyte cultures, while stimulation of these cells with IL-1β + TNFα resulted in the release of CCL2 into the culture medium (as measured by ELISA). Interestingly, at both doses CBD significantly decreased CCL2 release, with the greatest effect observed at 5 μM ($p \leq 0.001$).

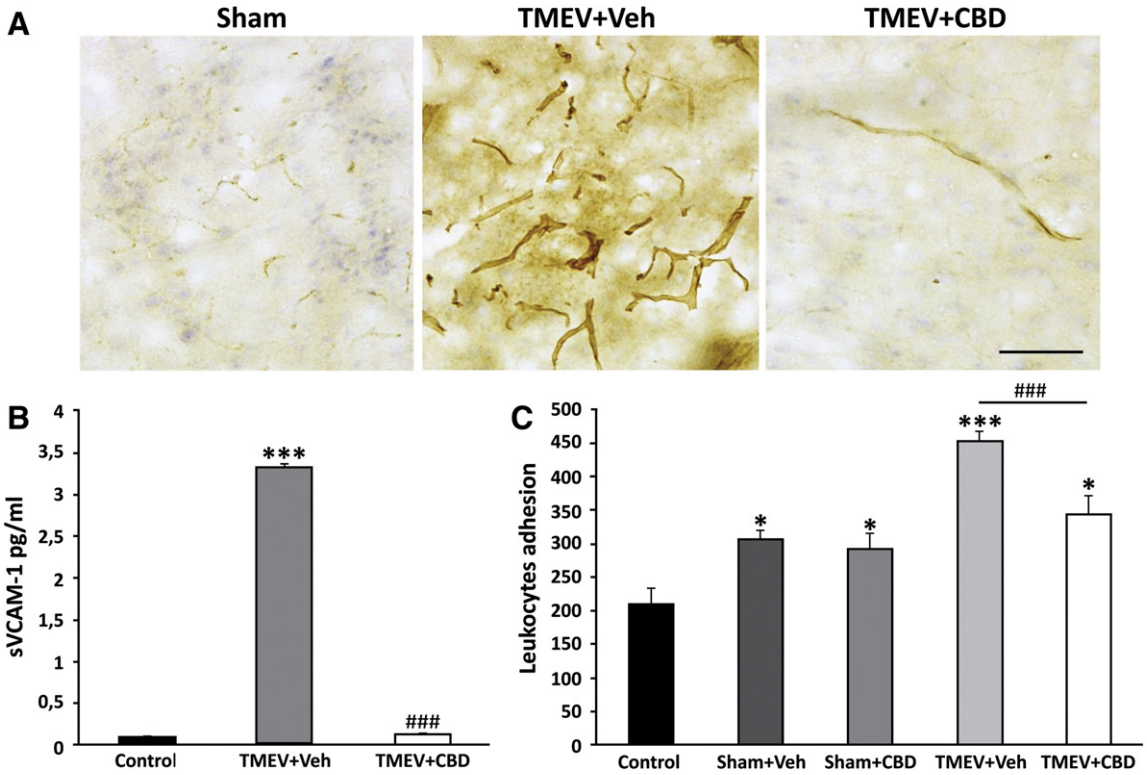

Fig. 1. CBD inhibits VCAM-1 production in response to TMEV infection and reduces leukocyte adhesion to endothelial cells. (A) Immediately after viral infection, sham and TMEV-infected mice were treated for 7 consecutive days with CBD (5 mg/kg) or the corresponding vehicle alone ($n = 6$ per group). Representative microphotographs of coronal brain sections (30 μm) immunostained for VCAM-1 showing positive staining in the blood vessels of TMEV-infected mice treated with the vehicle alone. Scale bar = 100 μm. (B) ELISA determination of sVCAM-1 levels in supernatants of endothelial cell cultures 20 h after treatment. TMEV-infected cells were pretreated for 1 h with CBD (1 μM). The results represent the mean ± SEM from 3 independent experiments performed in triplicate: ***$p \leq 0.001$ vs. control; ###$p \leq 0.001$ vs. TMEV-infected cells; Kruskal–Wallis ANOVA followed by Mann–Whitney U-test. (C) Brain endothelial cell monolayers were stimulated with TMEV virus for 6 h, and treated with vehicle or CBD (1 μM), and leukocytes from sham and TMEV-infected mice were stained with AM-calcein (5 μM), and then added to the endothelial culture for 20 h. Graph represents leukocyte adherence to the endothelial monolayer with respect to the control group ($n = 6$): *$p \leq 0.05$, ***$p \leq 0.001$ vs. control; ### $p \leq 0.001$ vs. leukocytes from TMEV-Veh animals; Kruskal–Wallis ANOVA followed by Mann–Whitney U-test.

CBD reduces leukocyte infiltration in the brains of TMEV-infected animals and it exerts anti-inflammatory effects in vivo

We investigated whether the regulatory effect of CBD on VCAM-1 and chemokine expression also altered neuroinflammation in TMEV-infected mice 8 days p.i. As revealed by H&E staining (Fig. 3A), intracerebral infection with TMEV promoted a strong infiltration of immune cells into the brain parenchyma at different sites, including the cerebral cortex and striatum. Moreover, CBD treatment decreased the infiltrate score (Fig. 3B) to that of sham infected animals, an effect that was particularly prominently close to the blood vessels where perivascular cuffing was observed in TMEV-infected animals. By contrast, no infiltrates were detected in mice administered CBD. In line with these findings, CBD treatment significantly reduced microglial activation in the cerebral cortex ($p \leq 0.01$), as revealed by Iba-1 staining (Figs. 4A, C). The microglia in TMEV-infected mice displayed the typical activated, ameboid morphology, whereas those isolated from CBD-treated mice exhibited a ramified morphology similar to that of the sham infected animals. Indeed, the immunosuppressive effect of CBD was accompanied by a decrease in IL-1β expression (Fig. 4B; $p \leq 0.05$), suggesting that the mode of action of CBD involves the restriction of immune cell transmigration to the CNS, as well as neuroinflammatory processes that occur after TMEV infection.

Long term immunomodulatory effects of CBD in the TMEV model

As CBD appears to be beneficial when administered in the induction phase of the TMEV-IDD model, we investigated whether the immunoregulatory actions of CBD have long term effects on the onset of symptoms, and on the spinal cord inflammation that occurs in the chronic phase of the disease. Mice were treated for 10 days with CBD immediately after infection with TMEV and on day 80, motor function was evaluated and histological and PCR analyses were performed. CBD treatment in the acute phase of the disease impaired disease progression in the chronic phase of TMEV-IDD, restoring both horizontal and vertical motor activities to that of the sham infected mice (Figs. 5A, B; $p \leq 0.05$). We next investigated whether CBD treatment also influenced the neuroinflammation observed in the chronic phase of the disease, as neurological dysfunction in the TMEV model has been traditionally associated with inflammation, demyelination and axonal loss in the white matter of the spinal cord (Ure and Rodriguez, 2002). The effects of CBD were accompanied by a reduction in microglial reactivity in the spinal cord (Figs. 5C, D; $p \leq 0.001$), and in the expression of the proinflammatory cytokines TNFα and IL-1β (Figs. 6A, B; $p \leq 0.001$), consistent with the immunomodulatory effects of CBD observed in the acute phase of TMEV infection.

Increased microglial reactivity is one of the events that occur at the encephalic level in the TMEV-IDD model, and it is accompanied by neurodegeneration and demyelination (Mecha et al., 2012b). CBD administration in the initial phase of TMEV infection significantly attenuated this process in the cerebral cortex during the chronic phase (Figs. 6C, D; $p \leq 0.05$).

Involvement of adenosine A_{2A} receptors in the effects of CBD in vivo and in vitro

Among the many pharmacological effects of CBD, some of its immunosuppressive actions may be independent of cannabinoid receptor

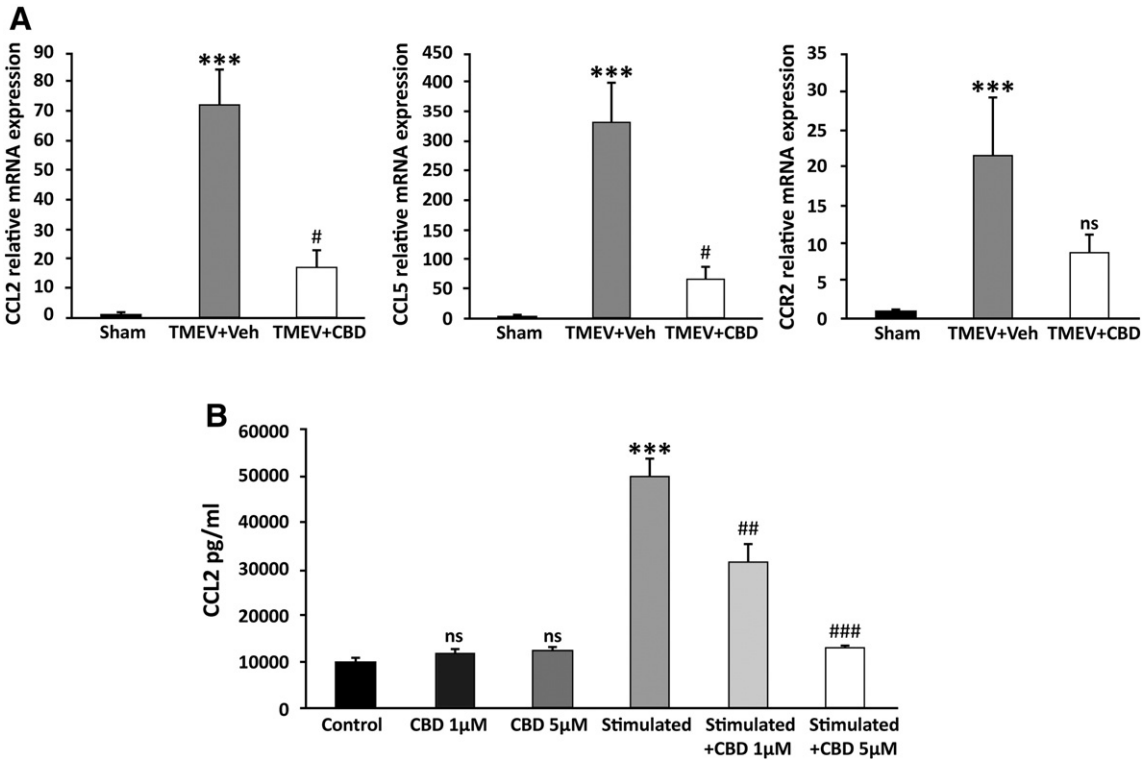

Fig. 2. CBD treatment decreases chemokine induction. (A) RT-PCR revealed that CBD treatment (5 mg/kg) decreased CCL2, CCL5 and CCR2 mRNA induction in the prefrontal cortex of TMEV-infected animals. The mRNA expression of each gene (n = 6 per group) was normalized to that of the 18S gene and the data represent the mean ± SEM: ***$p \leq 0.001$ vs. sham; #$p \leq 0.05$ vs. TMEV-Veh animals; Kruskal–Wallis ANOVA followed by Mann–Whitney U-test. (B) CCL2 levels were measured by ELISA in supernatants of cell astrocyte cultures 6 h after treatment, where IL-1β + TNFα-stimulated cells were pretreated for 1 h with CBD (1 or 5 μM). The results represent the means ± SEM from 3 independent experiments performed in triplicate: ***$p \leq 0.001$ vs. control; ##$p \leq 0.01$, ###$p \leq 0.001$ vs. stimulated astrocytes; Kruskal–Wallis ANOVA followed by Mann–Whitney U-test.

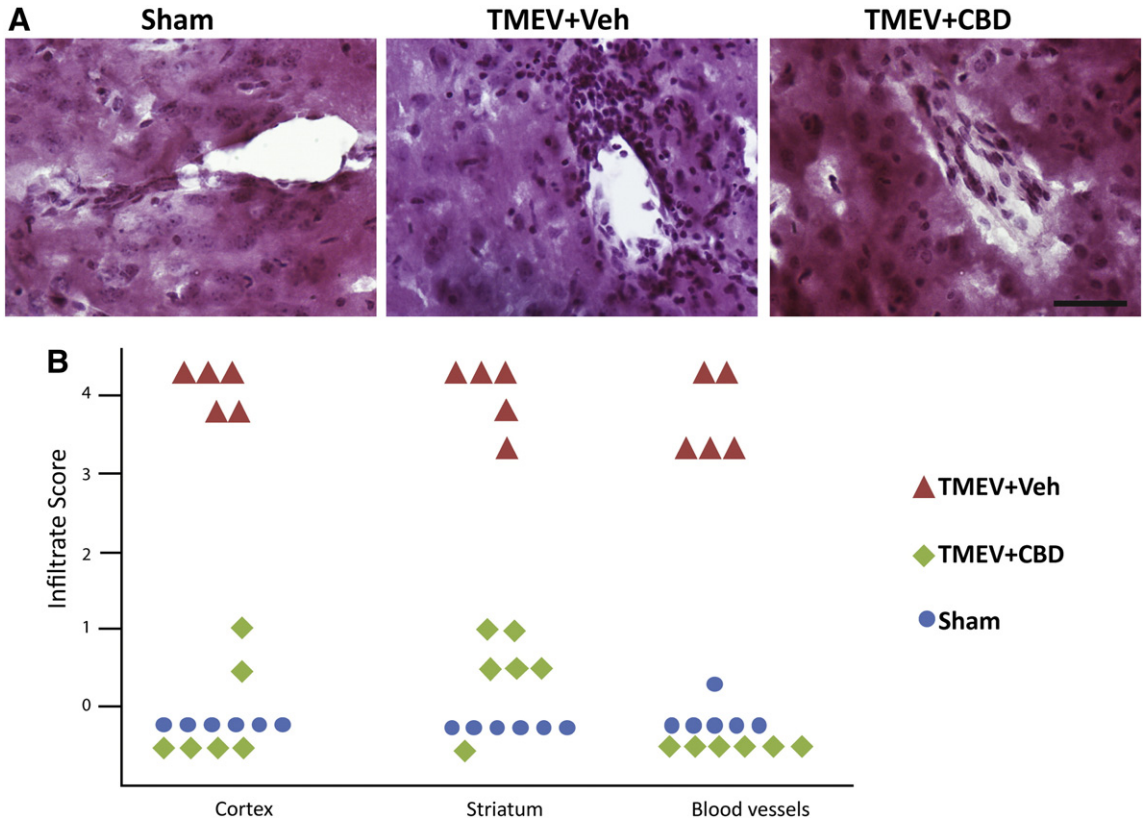

Fig. 3. CBD decreases leukocyte infiltration in the brains of TMEV-infected animals. Coronal brain sections (30 μm thick) were obtained at day 8 p.i. and stained with hematoxylin–eosin. (A) TMEV infection induced increases in leukocyte infiltration to several areas of the brain, particularly around blood vessels. Scale bar = 200 μm. (B) This effect was attenuated by CBD treatment (5 mg/kg), as indicated by the infiltrate score in the cerebral cortex, striatum and blood vessels.

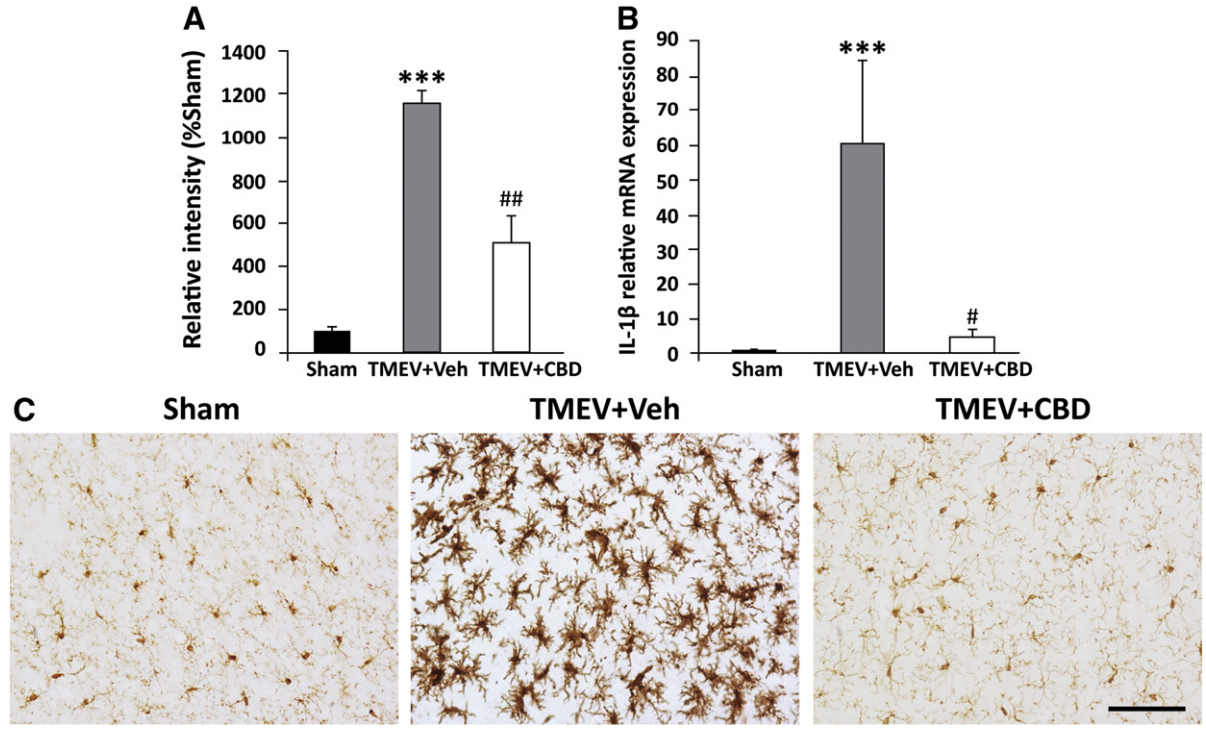

Fig. 4. CBD treatment attenuates the microglial response to TMEV infection. Coronal brain sections (30 μm thick) were obtained at day 8 p.i. and stained for Iba1. (A) Quantification of the percentage area occupied by microglia in the cerebral cortex per field ($n = 6$ animals per group): $^{***}p \leq 0.001$ vs. sham; $^{\#\#}p \leq 0.01$ vs. TMEV-Veh animals: one-way ANOVA followed by Tukey's test. (B) CBD treatment decreased IL-1β mRNA induction as determined by RT-PCR, in the prefrontal cortex of TMEV-infected animals, normalizing mRNA expression ($n = 6$ per group) to that of the 18S gene. Data represent the mean ± SEM: $^{***}p \leq 0.001$ vs. sham; $^{\#}p \leq 0.05$ vs. TMEV-Veh animals; Kruskal–Wallis ANOVA followed by Mann–Whitney U-test. (C) Representative microphotographs of Iba1 immunostaining in the above animals, showing morphological changes in the microglial cells of infected animals. Typical activated Iba1^{+} cells are evident in the cerebral cortex of TMEV-infected mice, while in sham and TMEV-infected mice treated with CBD (5 mg/kg), ramified, non-activated microglial cells are prominent. Scale bar = 100 μm.

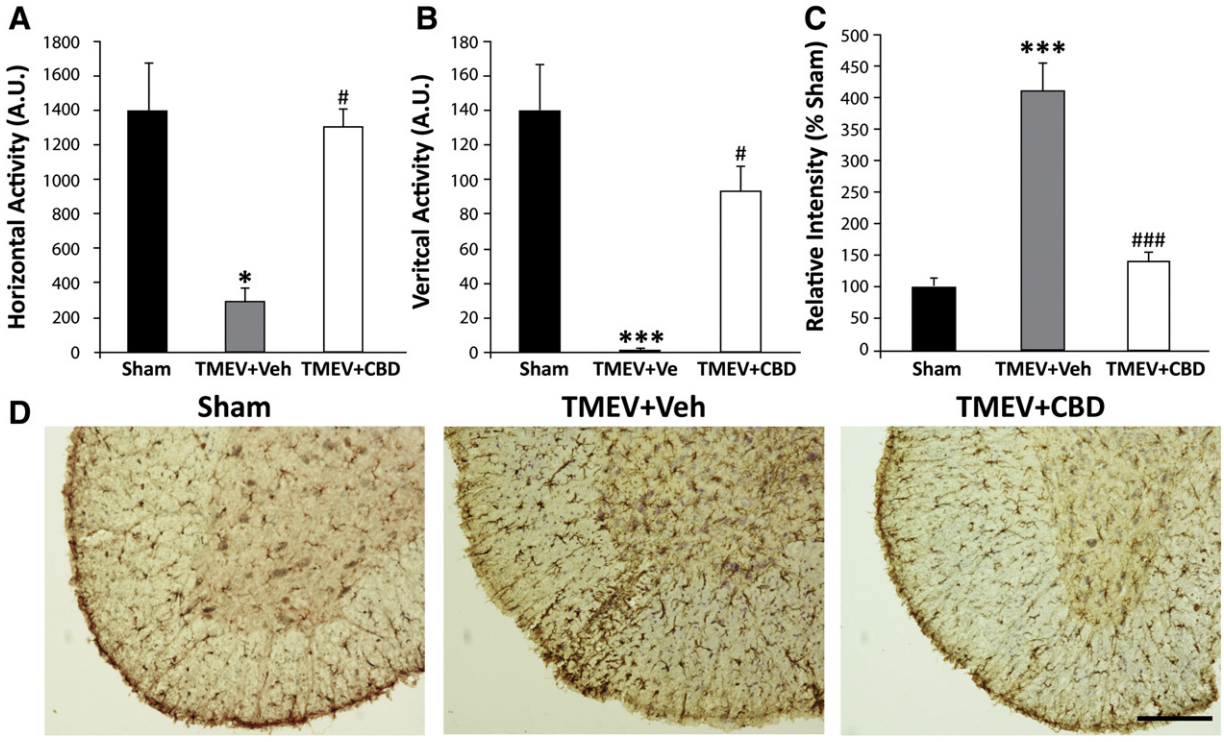

Fig. 5. CBD treatment for 7 days p.i. ameliorates motor deficits and decreases microglial reactivity in the chronic phase of TMEV infection. Sham and TMEV-infected mice were treated for 10 consecutive days with CBD (5 mg/kg) or the vehicle alone ($n = 6$ per group) immediately after viral infection, and they were analyzed on day 80 post-infection. Motor function was evaluated by measuring horizontal (A) and vertical (B) activities in the activity cage test, revealing a significant attenuation of motor deficits in the TMEV-IDD model following CBD treatment. Activity parameters were recorded for 10 min, and the results represent the mean ± SEM of 6 mice per group: $^{*}p \leq 0.05$, $^{***}p \leq 0.001$ vs. sham; $^{\#}p \leq 0.05$ vs. TMEV-Veh animals; one-way ANOVA followed by Tukey's test. (C) CBD treatment decreased microglial reactivity in the spinal cord of TMEV-infected animals when measured in the chronic phase of the disease. Quantification of the percentage area occupied by microglia in per spinal cord field, expressed as the mean ± SEM: $^{***}p \leq 0.001$ vs. sham; $^{\#\#\#}p \leq 0.001$ vs. TMEV-Veh animals; one-way ANOVA followed by Tukey's test. (D) Representative microphotographs of spinal cord sections from the aforementioned mice, cut at the cervical level and immunostained for microglia (Iba1). Scale bar = 100 μm.

activation and as CBD inhibits adenosine uptake mediated by the A_{2A} receptor, they may involve enhanced adenosine signaling (Malfait et al., 2000; Ribeiro et al., 2012). To explore this hypothesis, mice were administered with the selective A_{2A} antagonist ZM241385 at the time of TMEV infection and 30 min later, they were treated with CBD. In the brains of infected mice ZM241385 treatment attenuated some of the effects of CBD, including its inhibition of VCAM-1 expression (Fig. 7A) and of immune cell infiltration (Fig. 7B) and the reduction of Iba-1 immunoreactivity (Fig. 7C and Table 1). The administration of the antagonist did not have any effect in TMEV animals by itself. These observations suggest that the A_{2A} receptor is at least partially involved in the effects of CBD, as further supported by the dose–response effects of this A_{2A} antagonist on brain endothelial cell cultures in vitro. A_{2A} receptor antagonism with ZM241385 had a dose–effect blockade action, as at the dose of 5 μM (Table 2) completely blocked the inhibitory effects of CBD on sVCAM-1 release, determined by ELISA ($p \leq 0.001$), whereas the antagonism alone did not exert any effect in TMEV induced liberation of VCAM-1 (data not shown).

Discussion

In the present work we demonstrate that CBD counteracts some of the deleterious inflammatory responses to TMEV infection in the mouse brain. Sub-chronic treatment with CBD after TMEV inoculation of susceptible mice decreased the levels of several CNS mediators induced by viral infection, including VCAM-1, the chemokines CCL2 and CCL5, and the pro-inflammatory cytokine IL-1β. Similarly, this treatment decreased leukocyte infiltration and microglial activation, altering motor symptomatology and the neuroinflammation in the chronic phase of infection. These effects were also observed in cultured cells in vitro, and they appear to be mediated at least in part by the A_{2A} adenosine receptor.

The recruitment of activated immune cells through the endothelial cells of the BBB appears to be an essential step in the initiation of CNS inflammation in MS. In the brain and in endothelial cell cultures, CBD limited leukocyte migration to the nervous parenchyma in the acute phase of TMEV-IDD, probably by decreasing VCAM-1 expression, which reduced the adhesion of lymphocytes to endothelial cells and reduced infiltration in response to viral infection. These results are supported by our previous demonstration that impairing the upregulation of VCAM-1 following TMEV-IDD in vitro and in vivo attenuates the inflammatory responses and ameliorates the deficits produced (Mestre et al., 2009, 2011). CBD pretreatment also attenuates the high-glucose endothelial cell inflammatory response by decreasing VCAM-1 expression (Rajesh et al., 2007). It should be noted that the soluble fractions of VCAM-1 (sVCAM-1) increase in the cerebrospinal fluid and serum of MS patients in parallel with the progression of clinical disease (Correale and Bassani Molinas, 2003; Giovannoni et al., 1997; Hartung et al., 1995; Rieckmann et al., 1997). Accordingly, blocking the induction of this adhesion molecule appears to be essential to regulate the transmigration of activated cells from peripheral lymphoid tissues.

Chemokine release is another crucial step in the recruitment of inflammatory cells to the CNS. Chemokines are a family of low molecular weight, inducible, secreted, pro-inflammatory cytokines, which serve as potent chemoattractants for immune cells, thereby regulating their recruitment, trafficking and activation (Glass et al., 2003; Oppenheim et al., 1991). Pioneering studies in the TMEV-IDD model demonstrated that the increase in chemokine levels in the spinal cord is associated with the development of clinical symptoms (Hoffman et al., 1999) and

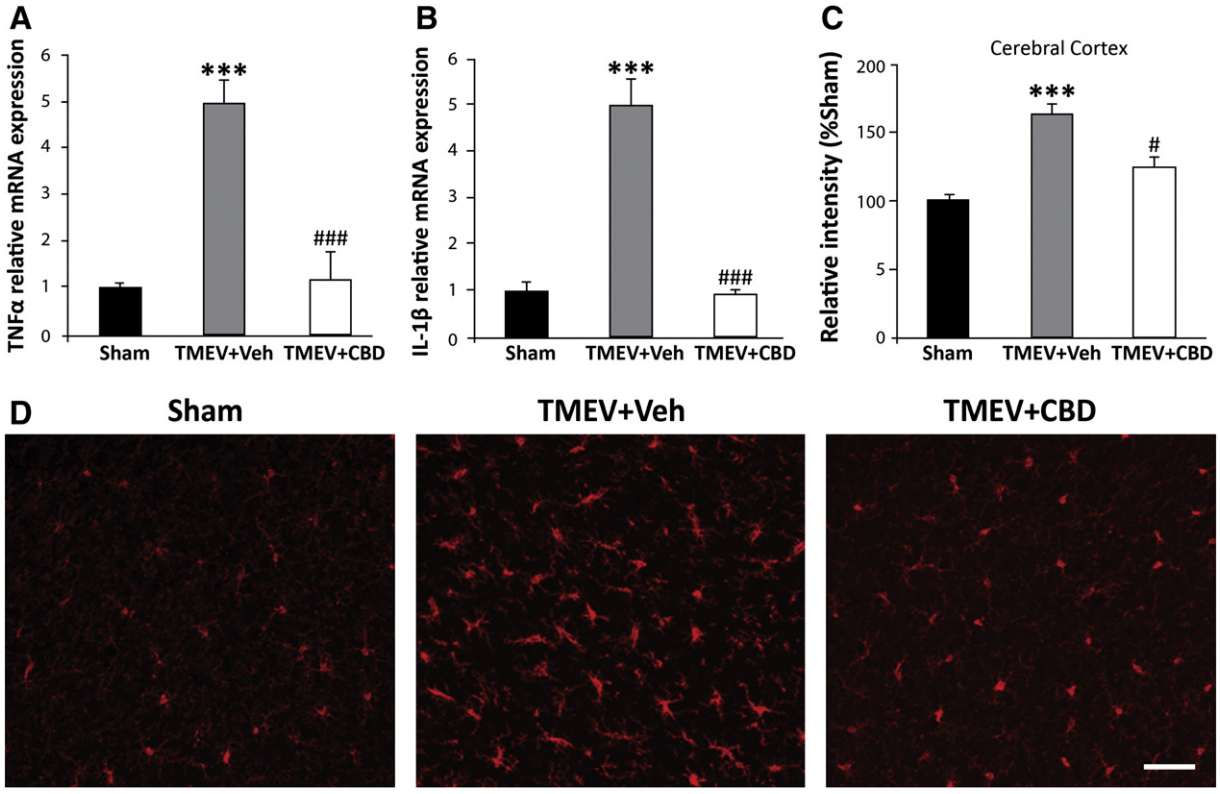

Fig. 6. CBD treatment exerts long-term effects that are evident in the chronic phase of TMEV-IDD, including decreased pro-inflammatory cytokine production in the spinal cord and reduced microglial reactivity in the cerebral cortex. CBD treatment (5 mg/kg) decreased TNFα (A) and IL-1β (B) mRNA induction in the spinal cord of TMEV-infected animals, as determined by RT-PCR normalizing mRNA expression ($n = 6$ per group) to that of the 18S gene. Data represent the mean ± SEM: ***$p \leq 0.001$ vs. sham; ###$p \leq 0.001$ vs. TMEV-Veh animals; Kruskal–Wallis ANOVA followed by Mann–Whitney U-test. Early CBD treatment also decreased microglial reactivity in the cerebral cortex of TMEV-infected animals when measured in the chronic phase of the disease. (C) Quantification of the percentage area occupied by microglia per cerebral cortex field, expressed as the mean ± SEM: ***$p \leq 0.001$ vs. sham; #$p \leq 0.05$ vs. TMEV-Veh animals; one-way ANOVA followed by Tukey's test. (D) Representative microphotographs of cerebral sections from the above mice, immunostained for microglia (Iba1). Scale bar = 100 μm.

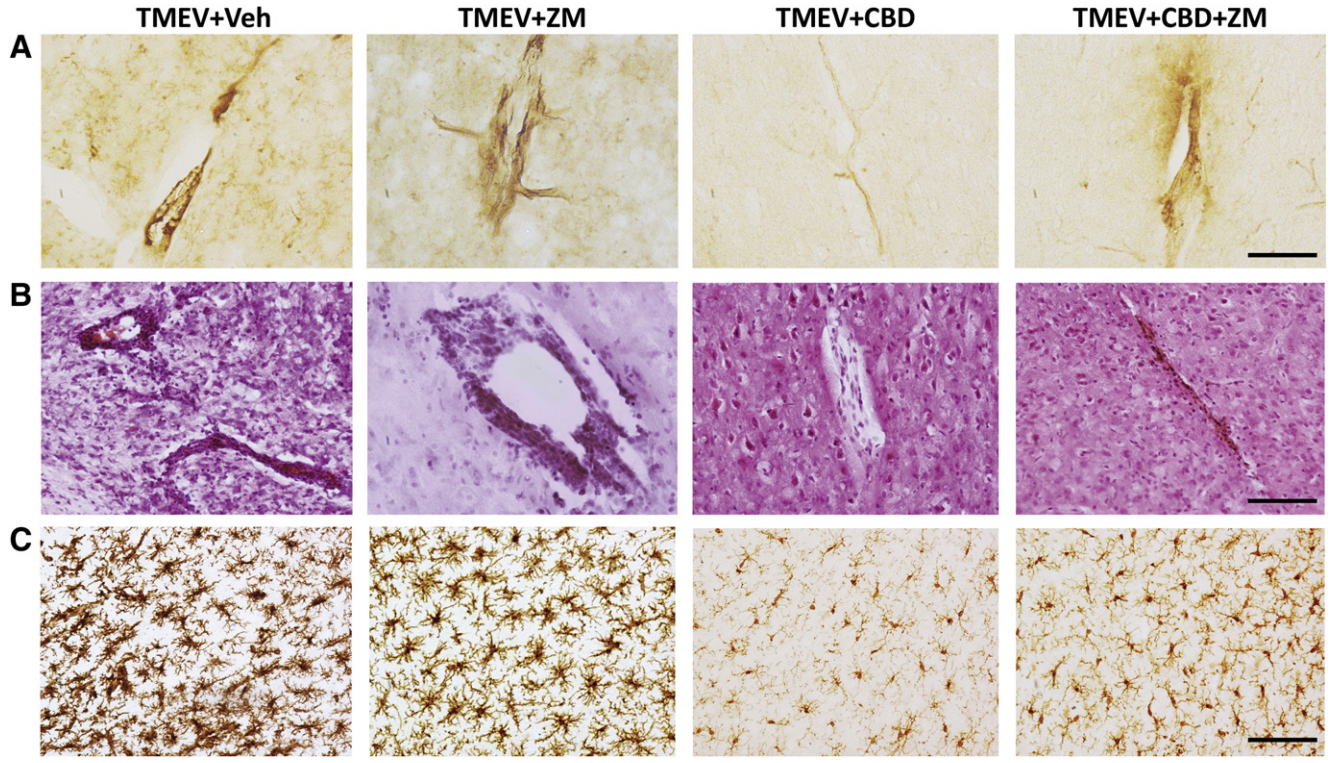

Fig. 7. A role for A_{2A} receptors in the mechanism of action of CBD in vivo. Immediately after viral infection, sham and TMEV-infected mice were treated for 7 consecutive days with the A_{2A} antagonist ZM241385 (5 mg/kg) 30 min before treatment with CBD (5 mg/kg) or the corresponding vehicle alone (n = 6 per group). Representative microphotographs of coronal brain sections (30 μm) showing VCAM-1 immunostaining (A), leukocyte infiltration (hematoxylin–eosin staining; B) and microglial activation (Iba1 immunostaining; C), show that A_{2A} antagonism did not exert any effect in TMEV infected mice, whereas it partially blocked the effects of CBD in the acute phase of TMEV infection. Scale bar = 100 μm.

subsequently, that CCL2 and CCL5 exhibit chemotactic activity for monocytes in the foci of active inflammation (Deshmane et al., 2009). Our results indicate that CBD limits chemokine production, as CCL2 and CCL5 expression decreased significantly in TMEV-infected mice treated with CBD. Moreover, our findings suggest an important immunoregulatory effect of CBD, which may limit the recruitment of lymphocytes to lesion sites by acting at both microglia and astrocytes. Indeed, anti-CCL2 treatment inhibits TMEV-IDD by decreasing mononuclear cell infiltration and CNS inflammation (Karpus et al., 2006). In MS patients, CCL2 is present in astrocytes and macrophages of active lesions, while CCL5 is present in lesions associated with the recruitment of T lymphocytes (Szczucinski and Losy, 2007). Here, the ability of CBD to inhibit VCAM-1 upregulation, together with its attenuation of the chemokine response, may explain the reduced immune cell infiltration seen in the brains of infected animals. In support of this view, it was previously demonstrated that CBD decreases leukocyte migration to the lungs in a model of acute inflammatory injury (Ribeiro et al., 2012), and CBD exerts anti-inflammatory effects at the vascular level following endotoxic shock induced by LPS (Ruiz-Valdepenas et al., 2011).

In addition to the effects of CBD at the level of the BBB, we found that this phytocannabinoid also interferes with the development of a deleterious inflammatory response in the brain of TMEV-infected animals in early disease stages. CBD treatment decreased microglial activation (as evident by immunohistochemistry) and the production of the pro-inflammatory cytokine IL-1β, mainly produced by macrophages/microglia. In TMEV-IDD, glial cells produce cytotoxic inflammatory mediators that contribute to the myelin loss and axonal damage characteristic of this model (Pope et al., 1996). Cannabinoids suppress the production of pro-inflammatory cytokines, including TNFα, IL-1β, IL-2, IL-6, IL-12 and IFN-γ, as well as mitogen-induced cell proliferation, migration, antigen presentation and trafficking to inflamed tissues (Croxford et al., 2005; Kozela et al., 2010; Rieder et al., 2012; Tanasescu and Constantinescu, 2010). Moreover, in a progressive EAE model, administration of CBD (5 mg/kg) during disease onset ameliorates disease severity by diminishing microglial cell activation and pathogenic T cell and immune cell infiltration in the spinal cord of immunized animals (Kozela et al., 2011). However, in a relapsing–remitting EAE model by inoculation of murine spinal cord homogenate, CBD at a dose range of 0.5 to 25 mg/kg for days 10 to 22 post-immunization did not alter clinical score while THC was

Table 1
A role for A_{2A} receptors in the mode of action of CBD in microglial cells in vivo. Quantification of the percentage area occupied by microglia in the cerebral cortex per field, using ZM241385 (5 mg/kg) 30 min before CBD treatment. The results represent the means ± SEM (n = 6 animals per group): $^{***}p \leq 0.001$ vs. TMEV + Veh animals; $^{###}p \leq 0.001$ vs. TMEV + CBD; Kruskal–Wallis ANOVA followed by Mann–Whitney U-test.

	Mean	SEM
TMEV + Veh	900.84	50.67
TMEV + ZM	918.00 ns	44.56
TMEV + CBD	102.33***	4.99
TMEV + CBD + ZM	191.45###	16.31

Table 2
A role for A_{2A} receptors in the mode of action of CBD in endothelial cells in vitro. sVCAM-1 levels in the supernatant of endothelial cell cultures were measured by ELISA 20 h after treatment. Endothelial cells were pretreated for 30 min with distinct concentrations of the antagonist ZM241385 (0.1, 1 and 5 μM), followed by CBD (1 μM) for 1 h before infection with TMEV. The results represent the means ± SEM from 3 independent experiments performed in triplicate: $^{***}p \leq 0.001$ vs. TMEV-Veh; $^{\#}p \leq 0.05$ vs. TMEV + CBD; $^{\#\#}p \leq 0.01$ vs. TMEV + CBD; Kruskal–Wallis ANOVA followed by Mann–Whitney U-test.

	Mean	SEM
TMEV + Veh	3.300	0.077
TMEV + CBD	0.133***	0.019
TMEV + CBD + ZM 0.1 μM	0.300 ns	0.022
TMEV + CBD + ZM 1 μM	1431#	0.037
TMEV + CBD + ZM 5 μM	3304##	0.172

effective (Maresz et al., 2007). Differences in the models of progressive MS vs. RRMS or administration schedules may account for the discrepant results among the labs. The data in the MS progressive models are consistent with the long-term anti-inflammatory effects of CBD treatment in the early stages of TMEV-IDD, which impaired early symptomatology in infected animals and decreased microglial activation and pro-inflammatory cytokine production in the chronic phase of the disease. It appears that attenuation of the early deleterious response of the peripheral and resident immune system has positive effects in the chronic phase of viral infection, resulting in less severe neurological deficits.

Although CBD exerts its effects through a variety of signaling pathways (reviewed in Izzo et al., 2009; Zuardi, 2008), adenosine A_{2A} receptors appear to be involved in some of its anti-inflammatory effects (Carrier et al., 2006). Treatment with ZM241385, a highly selective A_{2A} antagonist, attenuated many of the anti-inflammatory effects of CBD, including its effects on VCAM-1 expression (in vivo and in vitro), leukocyte infiltration and microglial activation in early disease stages. It was previous demonstrated that A_{2A} receptor activation attenuates inflammatory responses in a model of acute lung injury (Impellizzeri et al., 2011; Reutershan et al., 2007), while the inactivation of this receptor exacerbates EAE pathology (Yao et al., 2012) and blockade abrogates the anti-inflammatory effects of CBD (Ribeiro et al., 2012), consistent with our findings. Moreover, treatment with a specific A_{2A} receptor agonist decreases neutrophil and macrophage recruitment while reducing VCAM-1 immunoreactivity in a murine carotid ligation model (McPherson et al., 2001). In our experimental conditions, A_{2A} receptor blockade completely reversed the effects of CBD in endothelial cells, which may explain the observed attenuation of immune cell infiltration and microglia activation in the brain. While we cannot rule out the participation of other receptors in the mechanism of action of CBD, antagonists of CB1, CB2, PPARγ and TRPV1 failed to alter its effects in vitro (data not shown). Thus, it appears that inhibition of adenosine uptake and signaling via the adenosine A_{2A} receptor is one of the main mechanisms by which CBD exerts its anti-inflammatory effects in TMEV-IDD by modulating the VCAM-1 expression, although other actions of CBD such as inhibition of anandamide reuptake (Bisogno et al., 2001), CB2 inverse agonism (Thomas et al., 2007) or the inhibition of fatty acid amide hydrolase (FAAH), may also contribute to its anti-inflammatory effects.

There is a need for novel therapeutic strategies for MS and despite emerging evidence demonstrating the putative therapeutic effects of cannabinoids, their effective introduction into clinical use is strongly limited by unwanted psychotropic effects (Iuvone et al., 2009). However, in 2005 a mixture of THC and CBD administered as an oromucosal spray (Sativex®) was approved for the treatment of MS symptoms (Perras, 2005). CBD is well tolerated in humans, it has low toxicity and no psychoactive activity, and it exerts interesting immunomodulatory, antioxidant, neuroprotective (El-Remessy et al., 2008; Hampson et al., 1998; Iuvone et al., 2009) and oligoprotective effects (Mecha et al., 2012a), which may underlie its possible pleiotropic effects in neuroinflammatory pathologies. The present findings reveal an attractive therapeutic profile of CBD and suggest that CBD will have efficacy in controlling neuroinflammatory diseases such as MS. This compound can limit the harmful effects of an exacerbated inflammatory response, likely by increasing adenosine signaling, and prevent the development of secondary and irreversible damage.

Conclusions

In this study we present evidence that the non-psychotropic cannabinoid CBD has beneficial immunoregulatory actions in the TMEV-IDD model. CBD decreases the transmigration of blood leukocytes to the nervous parenchyma by downregulating the expression of VCAM-1 and the chemokines CCL2 and CCL5 and diminishes the activation of microglial cells in the early stages of the disease. Moreover, the treatment with CBD has long-term positive effects in TMEV-IDD and improves motor deficits associated with the disease. Finally, adenosine A_{2A} receptors are involved in the CBD-induced reduction of VCAM-1 by endothelial cells, defining a novel mode of action of CBD in neuroinflammatory processes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2013.06.016.

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